IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant	: Jan Zavada et.	Technology Center: 1600
Serial No.	: 09/807,949	Art Unit: 1643
Filed	: August 9, 2001	Confirmation No.: 9458
For	: MN Gene and Protein	Examiner: Christopher H. Yaen

REPLY BRIEF
37 CFR §41.41

MAIL STOP APPEAL BRIEF-PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The following Reply Brief for the above-identified application is in response to the Examiner's Answer mailed from the U.S. Patent and Trademark Office (PTO) on July 2, 2007. This Reply Brief is being filed within the two month period for response.

STATUS OF THE CLAIMS

Claims 1-30, 38, 40, 43 and 44 were previously cancelled. Claims 31-37, 39, 41 and 42, the only claims pending, are rejected in a Final Office Action mailed from the PTO on October 13, 2006, under two 35 USC 112, first paragraph rejections, from which this appeal is taken. The claims are identified in attached CLAIMS APPENDIX.

STATUS OF AMENDMENTS

No amendments have been filed since the October 13, 2006 Final Office Action from which this appeal is taken.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The subject claims, Claims 31-37, 39, 41 and 42, stand finally rejected under two 35 USC, first paragraph rejections.

First 35 USC 112, First Paragraph Rejection

Claims 31-37, 39, 41 and 42 stand rejected under 35 USC 112, first paragraph

> because the claimed "amino acid sequence" of the site still reads on a sequence as short as two amino acids derived from any of the claimed sequences listed in the Markush group. In other words, limiting the sequence by using Markush type language does not satisfy the lack of written description for a sequence of anything other than the sequence consisting of any one of SEQ ID NO: 10, 98-102 or 103. Again it is reiterated that **Appellant may overcome this rejection by amending the claims to indicate "the" sequence as opposed to "a sequence".**

[Final Office Action mailed from the PTO on October 13, 2006, paragraph bridging pages 3-4; emphasis added..] The Examiner in that rejection is referring to lines 4-5 of independent Claim 31 (as formatted in the attached Claims Appendix). The Examiner in referring to "'the' sequence" as opposed to "a sequence" is referring to the phrase "**an** amino acid **sequence** selected from the group . . . " at lines 4-5 of independent Claim 31.

The Examiner's Answer reiterates that rejection but includes SEQ ID NO: 50 within the Markush group. The Examiner's Answer also reiterates "that appellant may overcome this rejection by amending the claims to recite a peptide comprising 'the amino acid sequence'." [Examiner's Answer; page 5, 2nd full ¶; emphasis in original.]

Second 35 USC 112, First Paragraph Rejection "New Matter Rejection"

Claims 31-37, 39, 41 and 42 stand rejected under 35 USC 112, as a rejection of "new matter," based on a finding of lack of literal support in the Specification for a proviso added to the end of Claim 31:

> Applicant's [sic] have amended the claims to include a negative proviso limitation of "the non-MN-portion of said fusion protein or said fusion polypeptide does not contain a cell adhesion site". Applicant directs the examiner to page 21, lines 1-14 and page 69, lines 8-13 for support of this new limitation. However, the pages direct [sic] are drawn to the explanation of why the fusion protein would contain an additional binding site to which cells could potentially bind. There is no specific indication or disclosure that support a negative limitation or specific exclusion of fusion proteins missing a cell adhesion site as now currently claimed.

[October 13, 2006 Final Office Action, page 3, Section 5.] At page 5 of the Examiner's Answer, the Examiner repeats the finding of lack of literal support in the Specification for a proviso added to the end of Claim 31, stating that "Appellant is invited to point to clear support or specific examples of the claimed invention in the specification as-filed, by specifically pointing to further support for the negative limitation currently claimed." [Examiner's Answer, paragraph bridging pages 5-6.]

At pages 8-9, the Examiner further states:

> Appellants have amended the claims to include a negative limitation such that the non-MN portion of the fusion protein is to lack any cell binding sites in which MN would be capable of binding . . . . The support provided by the appellant does not provide those of skill in the art with any explicit or implicit support that all non-MN portions of the fusion protein should lack or be screened of potential binding sites.

ARGUMENTS

First 35 USC § 112, 1st ¶ Rejection is Improper: Use of Formal Markush Group Language

The first 35 USC 112, 1st ¶ rejection concerns the meaning of the phrase in Claim 31, lines 4-6: "wherein said site's amino acid sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 10 and 98-103. . . ."[1] The Examiner's Reply includes SEQ ID NO: 50 within the Markush group. The Applicants respectfully point out that the Markush group does not include SEQ ID NO: 50.

The Examiner's Answer reiterates that

> [t]he claims recite "an amino acid sequence" of any one of SEQ ID No: 50 [s/c], 10, or 98-10 [sic] as part of the of the invention. This reads on a fragment as small as two amino acid [sic] found within the sequence of SEQ ID No: 50 [s/c], 10, or 98-103."

[Examiner's Answer at page 3, 1st full ¶.]

The Examiner's Answer also reiterates "that appellant may overcome this rejection by amending the claims to recite a peptide comprising 'the amino acid sequence'." [Examiner's Answer, at page 5, 2nd full ¶; emphasis in original.]

Appellants respectfully repeat their argument presented in the Appeal Brief, submitting that the first 35 USC §112, 1st ¶ rejection of the October 13, 2006 Final Office Action is improper as it constitutes a rejection of standard Markush group claim language in independent Claim 31. Appellants respectfully submit that the phrase at issue from the first paragraph of Claim 31 – "wherein said site's amino acid sequence

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1. The Examiner's Answer refers at page 3 in the first full ¶ to SEQ ID NO: 1 being within the Markush group; however, that is an obvious error as SEQ ID NO: 1 is not an amino acid sequence but the full length cDNA (1522 bps) of the MN gene.

comprises an amino acid sequence selected from the group consisting of SEQ ID NOS.: 10 and 98-103 . . . " (emphasis added) – is formal Markush group claim language that inherently refers to the full-length amino acid sequence of one of a group of amino acid sequences identified by SEQ ID NOS., and would **not** be understood by ones of skill in the art to include within said group "a fragment as small as two amino acids . . . " from within one of the listed amino acid sequences, as argued by the Examiner.

Appellants respectfully question what is the antecedent of the Examiner's suggested phrase "the amino acid sequence. . . "? In Claim 31, the only antecedent could be "said site's amino acid sequence," but it is that amino acid sequence that comprises "an amino acid sequence selected from the group consisting of SEQ ID NOS: 10 and 98-103. . . ." Appellants are respectfully reluctant to change the "an" to a "the" under the circumstances, wherein the antecedent for "the amino acid sequence" is ambiguous, and such a phrase adds potential ambiguity to the independent claim. Appellants have and do remain respectfully open to alternative claim language with the equivalent intended meaning.

Appellants respectfully conclude that the invention as disclosed in the Specification as identified above was well within the Appellants' possession at the time the instant application was filed. Appellants respectfully request that the BPAI reverse the instant 35 USC §112, first paragraph rejection in view of the above remarks and the standard Markush group language at issue.

Second 35 USC § 112, ¶ 1 Rejection is Improper: Rejection of Inherent Characterization as New Matter

Appellants respectfully maintain that the second 35 USC 112, paragraph 1 rejection is improper as a "new matter" rejection of subject matter that is not "new matter" but instead constitutes a negative limitation clearly inherent in the Specification. Appellants respectfully maintain that the clarification of inherent characterization does not add new matter to an application. [See, for example, In re Smythe, 178 USPQ 279 (CCPA 1973).]

The Proviso at Issue

The following proviso, that is at issue, was added to the end of Claim 31 for greater clarity and particularity: "and wherein if said MN protein or said MN polypeptide is a fusion protein or a fusion polypeptide, the non-MN portion of said fusion protein or said fusion polypeptide does not contain a cell adhesion site." Appellants respectfully reiterate that that proviso at the end of independent Claim 31 only makes explicit what one of skill in the art would understand from the implicit teachings of the Specification.

The words of the proviso need not be the same as the wording in the Specification to comply with the written description requirement. "The test is whether the originally filed specification disclosure *reasonably* conveys to a person having ordinary skill that applicant had possession of the subject matter later claimed. . . ." [Ex parte Sorenson, 3 USPQ 2d 1462 (Bd. Pat App. & Interf. 1987) at page 1463; emphasis in the original.]

Appellants respectfully argue that the proviso at issue is well supported in the Specification and is intricately involved in the explanation of how the Appellants came to understand the nature of MN's cell adhesion site, that had been previously misunderstood as not being "closely related or identical" to the epitope for the M75 monoclonal antibody. [As explained throughout the Specification, for example, at least at page 62, lines 29-32, and at page 69, lines 8-13.]

Appellants respectfully submit that the two passages cited from the Specification in the Appeal Brief as providing support for the proviso of Claim 31 [at page 21, lines 1-14; and at page 69, lines 8-13], in addition to describing the use of MN fusion proteins, are also significant for the details concerning cell adhesion inhibition assays in the Pierschbacher and Ruohslahti 1984 article [cited in the Specification at page 21, line 13, and incorporated by reference at page 74, line 32 of the Specification]; and for the logical inference from the finding that the cell adhesion inhibition assay of Zavada et al. [Int. J. Oncol., 10: 857 (1997)] led to wrong conclusions because the GST-MN fusion protein used contained a second binding site. Appellants will discuss those points further below.

Appellants respectfully reiterate that the MPEP at §2163.07(a) makes it clear that a specification is interpreted according to what one of ordinary skill in the art would understand is supported both explicitly and implicitly, and that the claims may be amended accordingly without adding new matter:

> By disclosing in a patent application a device that inherently performs a function, operates according to a theory or has an advantage, a patent application necessarily discloses that function, theory or advantage, even though it says nothing explicit concerning it. The application may later be amended to recite the function, theory or advantage without

> introducing the prohibited new matter. *In re Reynolds* . . . 170 USPQ 94 (CCPA 1971); *In re Smythe* . . . 178 USPQ 279 (CCPA 1973).

According to the Specification, the implicit advantage of the representative MN full-length protein that was used successfully in Example 2 of the Specification to identify MN's cell adhesion site, over the inoperative GST-MN fusion protein used in Zavada et al. 1997 was that the representative MN full-length protein did not contain a second cell adhesion site. Appellants explain below how that advantage was inherent in the passages cited for support.

First Passage Cited in Appeal Brief: Specification at page 21, lines 1-14

In addition to referring to the use of MN fusion proteins in the assays of the invention, the first passage cited by Appellants for support stated in part:

> Further, peptides comprising the expected binding site can be synthesized. All of those products can be tested in cell adhesion assays, as exemplified below. [See, e.g., Pierschbacher and Ruoslahti, PNAS, 81: 5985 (1984); Ruoslahti and Pierschbacher, Science, 238: 491.]

[Specification, at page 21, lines 11-14.] The cited Pierschbacher and Ruoslahti 1984 article [copy attached] was incorporated in the Specification by reference [Specification, at page 74, line 32], and provides teachings concerning cell adhesion inhibition assays which support the proviso of Claim 31 and are exemplary of what was known conventionally in the art, many years before the subject application was filed. Although Appellants cited the passage referencing that Pierschbacher and Ruoslahti article in the Appeal Brief, Appellants would like to explain the particular relevance of that article to the negative proviso of Claim 31.

In particular, Pierschbacher and Ruoslahti 1984 teach that if a second cell adhesion site is present in a protein, it can alter the biological properties of a first cell adhesion site:

> The new cell attachment-promoting peptide sequences described here raise several intriguing possibilities regarding the cell attachment-promoting activity in fibronectin variants and other proteins. The sequence Pro-Arg-Gly-Asp-Val-Asp, which is predicted to occur in some fibronectin polypeptides but not in others as a result of alternative RNA splicings . . . , is active as a peptide, suggesting some fibronectin polypeptides may possess an additional cell attachment site. . . . **[S]ome of the differences that have been observed between the biological activities of fibronectins from different sources such as plasma and cultured cells . . . could relate to the presence of a different number of cell attachment sites**.

[Pierschbacher and Ruoslahti 1984; at page 5987, middle of right column; emphasis added.] From the conventional art of cell adhesion assays taught by Pierschbacher and Ruoslahti 1984, one of ordinary skill in the art would also know that a non-MN portion of an MN fusion protein could potentially contain a second binding site, since both Pierschbacher and Ruoslahti 1984 [e.g., Table 2 at page 5987] and the instant Specification [at page 69, lines 26-30] teach that cell adhesion site sequences as short as 3-4 amino acids could be found in many different proteins. From the explicit teachings of the Specification and from the teachings of what was conventionally known in the art, one of ordinary skill in the art would avoid using MN fusion proteins or MN fusion polypeptides having a second cell adhesion site that would potentially alter the biological activity of MN's cell adhesion site in the claimed assay. The terminal proviso of Claim 31 only makes that understanding explicit.

Second Passage Cited in Appeal Brief: Specification at page 69, lines 8-13

Appellants also respectfully contend that the added proviso only renders explicit a logical inference from the second passage cited by the Appellants from the Specification for support in the Appeal Brief. The second passage cited by Appellants for support occurs in the Specification at page 69, lines 8-13, and summarizes the reasons that the results from Example 2 using the representative full-length MN/CA IX protein were different from those of Zavada et al. 1997 which used the GST-MN fusion protein:

> There can be no doubt on the specificity of cell attachment to purified MN/CA IX+. It is abrogated by specific MAb M75, at a dilution 1:1000 of ascites fluid. This is a correction to our previous report in Zavada et al., Int. J. Oncol., 10: 857 (1997) in which we observed that MN/CA IX produced by vaccinia virus vector and fusion protein GST-MN support cell adhesion, but we did not realize that GST anchor itself contains another binding site, which is not blocked by M75.

[Emphasis added.]

That last sentence quoted immediately above from the Specification can be rephrased as: "If another binding site is present in the MN fusion protein used, then the cell adhesion inhibition assay does not work correctly." In the first statement of a syllogism, **A** might represent "**another binding site is present in the MN fusion protein used**" and **B**, "**the cell adhesion inhibition assay works correctly**":

If A, then ~ B

[If A, then not B]

Conditional Statement: "If another binding site is present in the MN fusion protein used, the cell adhesion inhibition assay does not work correctly."

The Law of Contrapositive states that if a conditional is true, so is its contrapositive. In this case, the contrapositive of the first (conditional) statement would be:

If ~ [~ B], then ~ A

Or: **If B, then not A**

Contrapositive: "If the cell adhesion inhibition assay works correctly, then another binding site is not present in the MN fusion protein used."

One of ordinary skill in the art wishing to use the claimed assay would presumably try to optimize the conditions of the cell adhesion inhibition assay as taught in the Specification, with as few uncontrolled variables as possible, and would therefore necessarily avoid the use of any MN fusion proteins with additional, non-MN-related binding sites. It is conventional in any experimental art that the more variables one includes in an experimental assay, the more controls are needed, and the more complicated the experiment and its interpretation become; therefore, it is conventional in any experimental art to **simplify an experimental assay as much as possible**. For that reason, Appellants respectfully but emphatically disagree with the statements in the Examiner's Answer at page 9 that

> [t]he support provided by the appellant does not provide those of skill in the art with any explicit or implicit support that all non-MN portions of the fusion protein should lack or be screened of potential cell binding sites. Even if it were scientifically sound to determine if there are in fact cell binding sites, alternative means could have been used to block those sites such that it would not interfere with the binding assay.

Appellants respectfully submit that ones of ordinary skill in the art, having been alerted by the Specification of the assay ending ineffectiveness of additional cell binding sites in the non-MN portions of MN fusion proteins, rather than selecting MN fusion proteins with additional cell binding sites and blocking such sites specifically, would instead avoid the use of such MN fusion proteins requiring additional experimentation, unless the intention were to infringe the claims by a klugy, inelegant get-around attempt. Why go to all that unnecessary effort?

Appellants further respectfully submit that there is other exemplary support in the Specification for Claim 31's proviso. For example, the Specification at page 63, lines 1-3 states: "It was concluded that ectopically expressed MN protein most likely participates in oncogenesis by intervention into normal cell-cell contacts. MN's binding site represents a potential target for which therapeutic agents can be designed."

As MN's binding site "represents a potential target for which therapeutic agents can be designed . . . ," one of ordinary skill in the art of drug design would not be motivated to include additional non-MN-related cell adhesion sites (that are potentially non-tumor-related) in any MN fusion protein used in the claimed screening assays. In fact, one of ordinary skill in the art of drug design would be motivated away from including such non-MN cell adhesion sites, in view of the teaching away of the identity of MN's cell adhesion site, that was caused by such a site in the GST-MN fusion protein

before that additional site was identified. If tumor specificity is an important aspect of MN's cell adhesion site, a second cell adhesion site could interfere with the biological activity of MN's distinctive, tumor-associated cell adhesion site. Why complicate the assay with cell adhesion sites derived from other proteins, that might only aid in the design of "drugs" that block the non-tumor-associated non-MN binding sites?

To summarize, the terminal proviso of Claim 31 only renders explicit the logical inference of the Specification at page 69, lines 8-13. Appellants respectfully conclude that no new matter was added by the addition of the negative proviso at the end of Claim 31 which renders what was implicit in the Specification explicit in independent Claim 31. For the reasons provided above, Appellants respectfully request that the second 35 USC §112, ¶1 rejection of all the pending claims be reversed.

Respectfully submitted,

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Dated: September 4, 2007

CLAIMS APPENDIX

Claim 31: A method of identifying an organic or an inorganic molecule that binds specifically to MN's cell adhesion site, to which site vertebrate cells adhere in a cell adhesion assay, wherein said site is within MN's proteoglycan-like domain, wherein said site's amino acid sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 10 and 98-103, said method comprising testing an organic or an inorganic molecule in a cell adhesion assay, wherein said cell adhesion assay comprises:

(a) allowing MN protein, which comprises said site, and/or MN polypeptide, which comprises said site, to bind to a substrate, to which substrate vertebrate cells do not bind;

(b) rinsing unbound MN protein or unbound MN polypeptide from said substrate;

(c) incubating the bound MN protein or the bound MN polypeptide with said organic or inorganic molecule, and with said vertebrate cells;

(d) rinsing unbound vertebrate cells from said bound MN protein or bound MN polypeptide; and

(e) if said organic or said inorganic molecule inhibits the adhesion of said vertebrate cells to said MN protein or to said MN polypeptide, identifying said molecule as specifically binding to said site;

wherein said site, and said MN protein or said MN polypeptide, are specifically bound by the M75 monoclonal antibody that is secreted from the hybridoma

VU-M75, which was deposited at the American Type Culture Collection under ATCC No. HB 11128, and wherein said MN protein or said MN polypeptide is encoded by a nucleotide sequence selected from the group consisting of:

(i) SEQ ID NO: 1;

(ii) nucleotide sequences that hybridize specifically under stringent hybridization conditions of 0.02 M to 0.15 M NaCl at temperatures of 50°C to 70°C to the complement of SEQ ID NO: 1; and

(iii) nucleotide sequences that differ from SEQ ID NO: 1 or from the nucleotide sequences of (ii) in codon sequence due to the degeneracy of the genetic code;

and wherein if said MN protein or said MN polypeptide is a fusion protein or a fusion polypeptide, the non-MN portion of said fusion protein or said fusion polypeptide does not contain a cell adhesion site.

Claim 32: The method of Claim 31 wherein said molecule is organic.

Claim 33: The method of Claim 31 wherein said molecule is inorganic.

Claim 34: The method of Claim 32 wherein said molecule is a protein or a polypeptide.

Claim 35: The method of Claim 34 wherein said protein or polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 137 and 138.

Claim 36: The method of Claim 34 wherein said polypeptide is selected from the group consisting of SEQ ID NOS: 137 and 138.

Claim 37: The method of Claim 31 wherein said organic or inorganic molecule, when in contact with a vertebrate preneoplastic or neoplastic cell that abnormally expresses MN protein, inhibits the growth of said cell.

Claim 39: The method of Claim 31 wherein said MN polypeptide is SEQ ID NO: 106.

Claim 41: The method of Claim 31 wherein said vertebrate cells are mammalian cells.

Claim 42: The method of Claim 31 wherein said vertebrate cells are human cells.

Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 5985–5988, October 1984
Biochemistry

Variants of the cell recognition site of fibronectin that retain attachment-promoting activity

(cell adhesion/extracellular matrices/collagen/synthetic peptides)

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Communicated by Clifford Grobstein, June 25, 1984

ABSTRACT **A tetrapeptide sequence, Arg-Gly-Asp-Ser, is the minimal structure recognized by cells in the large, adhesive glycoprotein fibronectin. We now have defined the structural requirements for this cell recognition site by testing several synthetic variants of the active tetrapeptide sequence. The conservative substitutions of lysine for arginine, alanine for glycine, or glutamic acid for aspartic acid each resulted in abrogation of the cell attachment-promoting activity characteristic of the natural sequence. However, in the position of the serine residue, some alterations were compatible with activity. Assay of peptides containing the structure Arg-Gly-Asp-X (where X = another amino acid residue) showed that an Arg-Gly-Asp-Val sequence predicted to be present in some, but not all, fibronectin molecules as a result of alternative RNA splicings could potentially create a second cell attachment site in those fibronectin polypeptide chains carrying that sequence. Other proteins with potentially active Arg-Gly-Asp-X sequences include several proteins that are known to interact with the cell surface. Among these are various types of collagens, thrombin, and discoidin, a slime-mold protein that may be involved in cell aggregation. The results presented here show that the arginine, glycine, and aspartic acid residues are absolutely required for the cell recognition, and that the surrounding amino acids may play a role in the expression of cell attachment activity in fibronectin and other proteins having this sequence. We suggest, based on these data, that this recognition mechanism may be common to a number of biological systems.**

Adhesion of cells is studied intensively because it is thought to be of prime importance for the control of cellular functions such as replication, motility, and differentiation. In recent years, rapid progress has been made in the analysis of the proteins that mediate the adhesion of eukaryotic cells to their extracellular matrices. Fibronectin has emerged as a prototype of such proteins.

Fibronectin interacts with other extracellular matrix macromolecules (1–3; for review, see ref. 4) and with a receptor(s) of an unknown nature at the surface of most eukaryotic cells. The interaction of fibronectin with cells results in the attachment and spreading of those cells on a surface covered with fibronectin (5–8; reviewed in ref. 4). We have determined the complete primary structure of the site in the fibronectin molecule that interacts with cell surfaces (9, 10) and have utilized this knowledge to synthesize peptides that reproduce the adhesive properties of fibronectin (11). Analysis of small synthetic peptides has shown that the recognition site for cells in fibronectin is carried by the sequence Arg-Gly-Asp-Ser (12). Peptides that contain this sequence promote cell attachment when insolubilized on a surface, and they also inhibit the attachment of cells to fibronectin when they are present in a soluble form (12). The Arg-Gly-Asp-Ser sequence is present in at least five other proteins, and at least one of these, the λ phage receptor protein of *Escherichia coli*, which has a Gly-Arg-Gly-Asp-Ser sequence in common with fibronectin, has cell attachment activity similar to that of fibronectin (unpublished data).

To define precisely the structural requirements for the recognition site, we assayed the cell attachment-promoting activities of a number of peptides with structures closely resembling the Arg-Gly-Asp-Ser peptide. We show here that the arginine, glycine, and aspartate residues cannot be replaced even with closely related amino acids, but that several amino acids can replace serine without loss of activity. The permutations in this fourth position add several proteins onto the list of proteins potentially capable of interacting with cell surfaces. The fact that some of these proteins are already known to interact with cell membranes may shed light on the nature of this interaction.

MATERIALS AND METHODS

Proteins and Peptides. Human fibronectin was isolated from freshly drawn plasma by using gelatin-Sepharose chromatography as described (3). Peptides were synthesized according to our specifications at Peninsula Laboratories (San Carlos, CA). When indicated, the peptides had a cysteine residue at the COOH terminus to facilitate coupling of the peptide to solid phases. The composition of the peptides was verified by amino acid analysis.

Cell Attachment Assays. Normal rat kidney cells (designated NRK cells) (13) were used for the assays. The peptides were tested for cell attachment-promoting activity by coupling them to microtiter wells through a protein coating as described (11, 12). Inhibition assays were carried out by including the soluble peptide in an assay where cells were attaching to microtiter wells coated with fibronectin by using a slight modification of the assay described earlier (12), since inhibition by the peptides was found to be dependent upon cell number and time of incubation. Briefly, NRK cells were released from nearly confluent cultures with trypsin (Sigma), washed with a solution containing soybean trypsin inhibitor (Sigma), and plated at a density of 8×10^3 cells per well in fibronectin-coated wells (2 μg/ml) already containing peptide. After 30 min, nonbound cells were washed away, and attached cells were fixed, stained, and counted. Incubation media for these experiments consisted of Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with glutamine/penicillin/streptomycin at 2 mM, 100 units, and 100 μg/ml, respectively (Irvine Scientific) and bovine serum albumin at 2 mg/ml (Sigma).

Sequence Search. Computer searches through published protein sequences were conducted by the National Biomedical Research Foundation (Georgetown University, Washington, DC) using the program SEARCH.

RESULTS

Two assays were used to study the contribution of each of the amino acids in the Arg-Gly-Asp-Ser sequence toward the

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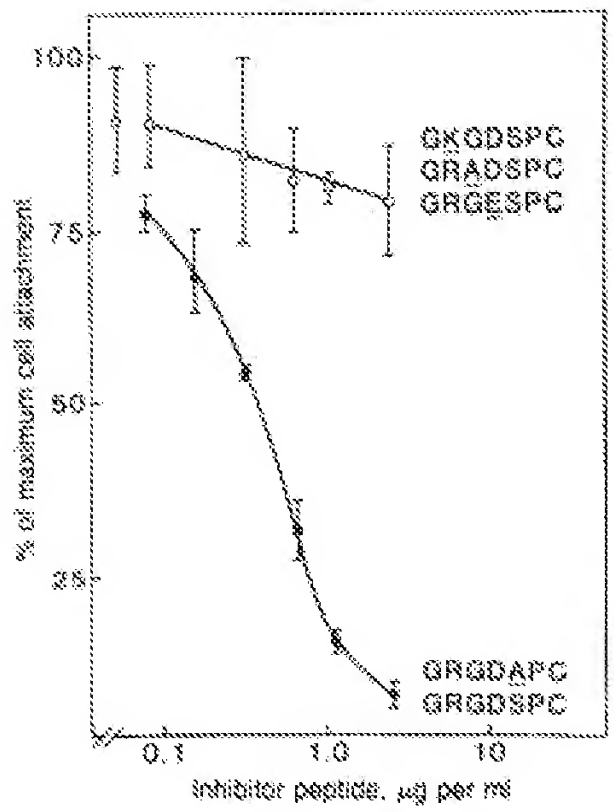

FIG. 1. Inhibition of attachment of NRK cells to immobilized fibronectin by synthetic fibronectin peptides. Microtiter wells were coated with human plasma fibronectin, and the attachment of NRK cells was assessed after incubation in the presence of various concentrations of soluble peptides. The attached cells were fixed, stained, and counted using an Artek cell counter. Data are represented as the mean of three experiments (± the range). Maximum attachment was about 50% of the cells plated.

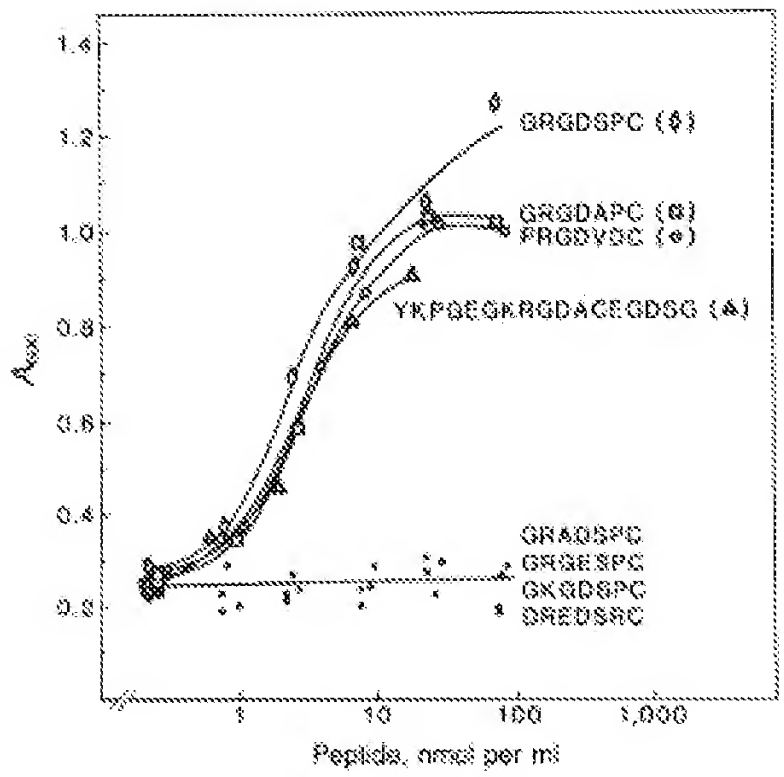

FIG. 2. Attachment of NRK cells to an immobilized synthetic fibronectin peptide and its variants. The synthetic peptides were assayed for their ability to promote the attachment of NRK cells by first attaching the peptides through the heterobifunctional cross-linker *N*-succinimidyl 3-(pyridyldithio)propionate (Sigma) to rabbit IgG that had been immobilized on polystyrene. The attachment assay was then carried out as described (14) with freshly trypsinized NRK cells. After a 1-hr incubation at 37°C, those cells that had attached were fixed, stained, and quantitated by using a vertical pathway spectrophotometer (Flow Laboratories). Maximum attachment was about 80–90% of the cells plated.

cell attachment-promoting activity. First, a set of five peptides, each differing by only one amino acid, were compared in terms of their capacity to inhibit attachment of cells to a substrate coated with intact fibronectin. In addition, the ability of each of the synthetic peptides described here to interact with cells was determined directly by using a cell attachment assay as described (11, 12). The results of these two experiments are shown in Figs. 1 and 2, respectively, and are summarized in Tables 1 and 2.

The two assays gave concordant results. Regarding the glycine in the active sequence, we previously had shown that substitution of this amino acid with the bulky valine residue abrogates the cell attachment activity (12). Results in Figs. 1 and 2 show that the introduction into this position of alanine, which has a relatively small side chain, or of glutamic acid also produces an inactive peptide. Similarly, either of the conservative substitutions of the charged amino acids arginine and aspartic acid with lysine and glutamic acid, respectively, resulted in a peptide that had no detectable cell attachment-promoting activity.

Earlier results also suggested that at least some variation in the position occupied by serine is compatible with activity because, when this residue was replaced with the closely related cysteine residue, full activity was retained. Therefore, we tested a number of peptides with the structure Arg-Gly-Asp-X, in which X stands for another amino acid residue. These peptides were designed either after sequences in other proteins that are capable of interacting with cells (see below), such as collagen and thrombin, or after sequences present elsewhere in the fibronectin molecule (see Table 2). The results presented in Fig. 2 (and compiled in Tables 1 and 2) show that, in addition to the previously reported cysteine, at least threonine, alanine, and valine can occupy this position with retention of activity. In contrast, using two collagen peptides in which lysine or hydroxyproline substitute for the serine, no activity could be demonstrated.

DISCUSSION

Each of the three amino acids in the sequence Arg-Gly-Asp appears to be integral to the site recognized by cells through the cell-surface structures (receptors) that bind fibronectin. This conclusion is based on the complete lack of activity that was observed with synthetic peptides in which substitutions were made by using the most closely related amino acid (lysine for arginine, alanine for glycine, and glutamic acid for aspartic acid) to replace one of the original three. Since these conservative substitutions give inactive peptides, it seems reasonable to conclude that other, less closely related amino acids would also render the sequence inactive, as is the case when asparagine replaces aspartic acid in the sequence (12) or when valine (12) or glutamic acid are substituted for the glycine residue.

The role played by the amino acids flanking the Arg-Gly-Asp sequence is less clear. We have shown earlier that the glycine residue, which in fibronectin is on the NH_2-terminal side of the arginine residue, imparts improved activity to those peptides containing it (12). On the other hand, fibrinogen, which has the Arg-Gly-Asp-Ser sequence in common with fibronectin, is not active in promoting the attachment of NRK cells, even though a nonapeptide modeled after the

Table 1. Activity of structural analogues of the cell attachment-recognition sequence of fibronectin in the cell attachment assays

Peptide	Interaction with cells
Gly-Arg-Gly-Asp-Ser-Pro-Cys	Active
Gly-Lys-Gly-Asp-Ser-Pro-Cys	Inactive
Gly-Arg-Ala-Asp-Ser-Pro-Cys	Inactive
Gly-Arg-Gly-Glu-Ser-Pro-Cys	Inactive
Gly-Arg-Gly-Asp-Ala-Pro-Cys	Active

Table 2. Proteins containing potentially active cell attachment sequences

Sequence*	Protein	Ref.
RGDS	α-Lytic protease, *Myxobacter* 495	16
	Testis-specific basic protein, rat	17
	Fibrinogen α chain, human	18
	λ receptor protein, *E. coli*	19
	Coat and membrane polyprotein, Sindbis virus	20
	Viral protein 1, foot-and-mouth disease virus	21
RGDA	Collagen αIII, bovine and human	22
	Thrombin, bovine and human	23
	Discoidin I, A chain, *Dictyostelium discoideum*	24
	Vitellogenin I precursor, fruit fly	25, 26
	3-Hydroxyacyl-CoA dehydrogenase, pig	27
	Collagen α2(I), chicken	22
RGDT	Collagen α2(I), human	28
	F1 protein, human influenza A virus (two strains)	29, 30

*The one-letter amino acid code (15) is used.

fibrinogen amino acid sequence around the Arg-Gly-Asp-Ser tetrapeptide in fibrinogen is active (12). These observations suggest that the surrounding sequences in an intact protein may either enhance or suppress the activity of the cell attachment sequence. Possible mechanisms for such modulation of activity include folding of the tetrapeptide in such a way as to make the Arg-Gly-Asp sequence more (or less) available for the cell-surface receptor or to cause steric hindrance of this interaction by a nearby amino acid. Both of these mechanisms may contribute to the effects we have observed when replacing the serine residue with other amino acids.

Several amino acids can occupy the position of serine in the fibronectin sequence without substantially changing the activity of the resulting peptide as long as the Arg-Gly-Asp sequence remains intact. We have so far tested six substitutions and found that the ones with threonine, alanine, valine, and cysteine are active. Two peptides, each modeled after sequences occurring in collagen chains (28, 31, 32) and containing a lysine or hydroxyproline residue at the serine position, were inactive, suggesting that there are restrictions concerning which amino acids can occupy this position. The latter peptides differ from the active peptides with regard to several other amino acids surrounding the Arg-Gly-Asp sequence. However, since all of the Arg-Gly-Asp-Ser-containing peptides that we have tested have been active in promoting cell attachment regardless of surrounding sequences, it is likely that it is the substitution of serine that prevents the expression of this activity in these peptides. A nonapeptide from thrombin (33) that contains the sequence Arg-Gly-Asp-Ala was active, as would be predicted from the results described above for the modified fibronectin peptides, suggesting that surrounding sequence can be significantly altered with retention of activity, at least within the peptides.

The new cell attachment-promoting peptide sequences described here raise several intriguing possibilities regarding the cell attachment-promoting activity in fibronectin variants and other proteins. The sequence Pro-Arg-Gly-Asp-Val-Asp, which is predicted to occur in some fibronectin polypeptides but not in others as a result of alternative RNA splicings (34), is active as a peptide, suggesting that some fibronectin polypeptides may possess an additional cell attachment site. At this point our results should be interpreted with caution, since we have not yet shown that this sequence is active when integrated into the protein. However, some of the differences that have been observed between the biological activities of fibronectins from different sources such as plasma and cultured cells (4, 35–37) could relate to the presence of a different number of cell attachment sites. On the other hand, the sequence from the extreme COOH terminus of the fibronectin molecule, Asp-Arg-Glu-Asp-Ser-Arg-, had, as was predicted from earlier results (12), no activity.

Various collagen chains contain Arg-Gly-Asp-X sequences (28, 31, 32), and in some of these, the X position is occupied by a residue that we have shown to be compatible with cell attachment activity. The presence of the potentially active sequences in collagens is of particular interest for two reasons. First, collagens have been shown to mediate cell attachment independent of fibronectin (38–40); second, variations of the tetrapeptide sequence are especially abundant in collagens. Such sequences are repeated at least three times along the α2 chain of type I collagen, and several copies are present in other collagen types that have been sequenced (see ref. 22). These observations suggest that the Arg-Gly-Asp sequence may be involved in the cell attachment-promoting activity of collagen. However, as yet we have no direct proof of this, and it may well be that the rigid triple helical structure of native collagen suppresses the activity of the Arg-Gly-Asp sequences. Cell lines have been described that attach to fibronectin but not to type I collagen (7, 41–43), suggesting also that other mechanisms may be involved in the attachment of cells to collagen. In any event,

Table 3. Selected list of sequences homologous to the cell attachment site of fibronectin

Peptides	Sequence*	Ref.	Interaction with cells
Fibronectin			
Cell attachment site, all fibronectins	A-V-T-G-R-G-D-S · P-A-S · S-K	3	Active†
Second cell attachment site (?), variant rat fibronectin	G-H-V-F-R-G-D-V · D-Y-H · L-Y	34	Active†
Phosphorylation site, bovine fibronectin	V-Q-A-D-R-E-D-S · K-E	44	Inactive
Collagen			
α2(I)	A-P-G-L-R-G-D-T · G-A-T · G-R	22	Active
α2(I)	P-Q-G-I · R-G-D-K · G-E-P · G-E	22	Inactive
α1(IV)	D-X-G-S-R-G-D-P‡ · G-T · P‡ · G-V	31	Inactive
Thrombin	G-E-G-K-R-G-D-A · C-E-Q · D-S	33	Active†

*The one-letter amino acid code (15) is used. Sequences flanking the actual synthetic peptides tested have been included for comparison.
†The actual synthetic peptide tested and the results obtained with that peptide are shown in Fig. 2.
‡4-Hydroxyproline.

our results point to new ways of exploring the interaction of cells with collagens.

A variety of other proteins contain the Arg-Gly-Asp sequence and, therefore, could have the same capacity as fibronectin to interact with cell surfaces. A selected list of such proteins is presented in Table 3. We have found that one protein that we studied because it possesses this sequence, the λ phage receptor of *E. coli*, actively promotes the attachment of NRK cells (unpublished data). This confirms the possibility that some of the other proteins on this list could be active. A particularly interesting candidate is discoidin Iα (24, 45, 46), a slime-mold protein believed to be involved in cellular aggregation.

The nature of the cellular receptor for fibronectin is unknown. If indeed the fibronectin-cell interaction is a manifestation of a widely distributed recognition mechanism, the critical aspects of the structure that interacts with the Arg-Gly-Asp sequence also must be conserved. Thrombin, which has an Arg-Gly-Asp-Ala sequence, binds to many types of cells by interacting with apparent cell-surface receptors (47). The Arg-Gly-Asp-Ala sequence is near the catalytic serine in thrombin and could be involved in cell-surface binding (47). Perhaps these receptors are related to the fibronectin receptor, which has proven elusive in spite of much study devoted to its identification.

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